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14. ABSTRACT
In Year 3 of the project we have completed Task 3 and began the work in Task 4 (as per approved Statement of Work). As a result, we have rescued, propagated and characterized a panel of adenovirus vectors that are now ready for the in vivo studies that will conclude the project. To facilitate the monitoring of tumor growth and regression in these studies, we have also developed cell lines expressing dual reporters that are suitable for non-invasive in vivo imaging of tumors.

15. SUBJECT TERMS
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Table of Contents

INTRODUCTION:

The scope of the proposed work is to develop novel Ad vectors, which will be targeted to vasculature of prostate tumors via genetic incorporation into their capsid of the recently identified vasculature endothelium-specific peptides. Additionally, tumor selectivity of these vectors is to be further improved by limiting the expression of the therapeutic transgene carried by the vectors, to endothelial cells of tumor vasculature. These double-targeted viruses are to be able to selectively infect blood vessels within the tumors and destroy the blood supply to tumors by locally expressing "suicide" transgene.

REPORT BODY

In our previous progress report (Year 2), we presented the work accomplished in Tasks 2 and 3 of the project. At the time of the report, Task 2 was completed while Task 3 was not. Therefore, the work in the Year 3 of the project started with the continuation of experiments described in Task 3. The present report describes the completion of Task 3 and the work towards the goals of the final Task 4.

Description of work:

Task 3. Generation and preliminary characterization of Ad vectors incorporating the flt-1 promoter-controlled dual expression cassette and the fibers modified with targeting peptides.

Experiments and results:

Year 2 of the project ended with the generation of eight recombinant Ad genomes, which were modified in two different ways. First, the E1 region within these genomes was deleted and replaced with the expression cassettes containing an open reading frame of a genetic fusion of HSV TK and firefly luciferase ("TL" for short) under transcriptional control of either the cytomegalovirus immediate-early promoter (control vectors) or human Flt-1 promoter (vasculature endothelium-specific). Second, the wild type Ad5 fiber gene within these genomes was replaced with the fiber genes modified by genetic incorporation of the previously identified prostate vasculature-specific peptide ligands. In addition, these fiber genes were mutated to prevent their products from binding to both the well established Ad5 receptor (CAR) (6), and heparan sulfate glycosaminoglycans (HSG), which are currently viewed as potential receptors for Ad5 (4, 7-9). Of note, the described fibers were tested in the fiber trans-complementation assay and proved to incorporate into Ad virions efficiently. In addition, two genomes each carrying one of the two expression cassettes and the wild type fiber gene were designed for control purpose.

Therefore, our next goal was to use the designed Ad genomes (above) to rescue the viruses. Because the viruses to be rescued were expected to contain mutated fibers and, thus, unable to use the natural mechanism of cell infection, the rescue was done using the previously established cell line, 293/F28 (1, 2) that expresses the wild type Ad5 fiber. Transfection of these cells with the designed Ad genomes yielded mosaic virions that incorporated both the wild type and the modified fibers. The presence, of the wild type fiber made these vectors infectious and facilitated further amplification in 293/F28 cells. The last round of amplification was supposed to be done in regular 293 cells (no wt fiber expression) to yield virions equipped with targeted fibers only.

By the time the project reached this point, the data that we have generated in another on-going project, which also involves Ad targeting, made us think that the idea of mutating the putative HSG-binding site within our fiber constructs was wrong. The troubling news was that the double-mutated, ligand-modified fibers that have been designed in that other project and (importantly!) confirmed to bind the target receptor (Her2), proved to be very inefficient in directing Ad virions

to this receptor. In contrast, the viruses equipped with the fiber constructs that contained only one mutation (in the knob) showed high levels of target cells transduction. This observation made us revisit the entire idea of mutating the fiber shaft as a means to ablate undesirable binding of Ad vectors to HSG. We realized that the site of the mutation previously proposed to ablate this binding (8) is located immediately downstream to the so-called "*hinge*" region within the shaft domain. This hinge is known to give the fiber its flexibility that is a key structural feature of the protein, because it allows a CAR-anchored Ad virion to form the secondary contact with the cell and that in turn triggers Ad internalization. It has been previously shown that the fibers with mutated hinge yield Ad vectors with poor infectivities (10). These considerations led us to the conclusion that the use of Ad vectors with double-mutated fibers in the *in vivo* studies in Task 4 would most likely yield negative results with respect to both the specificity and efficacy of gene transfer. Should this happen at such a late time point in the project, we would not be able to remake the vectors and redo the experiments in animals because there would not be any time and money left. Therefore, we decided to remake these vectors using the fiber genes with only one mutation.

To further support that decision, we would like to reference the study by Kritz *et al*. (3) published earlier this year, which has demonstrated that the targeted Ad vectors containing the fiber proteins with the mutated shaft domain near the hinge region are ineffective for cell retargeting strategies.

Having made this decision, we went back and redesigned all the fiber genes and the shuttle vectors, remade the rescue plasmids containing Ad genomes and these modified fiber genes. A total of eight Ad genomes (Table 1) have been remade.

Next, the Ad vectors were rescued and propagated using the fiber-complementing cell line 293/F28 (as described above). Because this work took several months and caused a significant delay in the project, and also consumed extra funds, at this juncture we decided to limit the total number of vectors to be used further in the project to a total of four (shown by gray shading in Table 1). These selected Ad vectors were then re-packaged by an additional round of propagation that was done in 293 cells. Specifically, each of the vector configurations amplified previously in 293/F28 cells was used to infect 2×10^9 293 cells. The re-packaged virions were then purified by equilibrium centrifugation in CsCl gradients yielding the amounts shown in Table 1. To confirm the identities of these Ad vectors, DNA sequencing of the modified fiber genes was done.

Table 1. Summary of Ad vector constructs re-made in Year 3 of the project. Vectors shown by gray shading will be used in the *in vivo* experiments in Task 4. In addition, two control Ad vectors, Ad5CMV-TL.F5 and Ad5Flt-TL.F5, each equipped with the wild type fibers have been produced.

Vector	Promoter that drives the reporter gene	Peptide ligand and its position within the fiber	Total yield in the final preparation (vp)
Ad5CMV-TL.PB40-1.7	CMV	*GRRAGGS* in the HI loop	1.6E+13
Ad5Flt-TL.PB40-1.7	Flt	*GRRAGGS* in the HI loop	1.6E+13
Ad5CMV-TL.PB40-1.12	CMV	*VAGGVAGGA* in the HI loop	1.8E+13
Ad5Flt-TL.PB40-1.12	Flt	*VAGGVAGGA* in the HI loop	1.3E+13
Ad5CMV-TL.PB40-1.1	CMV	*SMSIARL* in the HI loop	
Ad5Flt-TL.PB40-1.1	Flt	*SMSIARL* in the HI loop	
Ad5CMV-TL.Fc3.4	CMV	*VSFLEYR* at the carboxy terminus	

Ad5Flt-TL.Fc3.4	Flt	*VSFLEYR* at the carboxy terminus	

In addition, the efficacy of incorporation of the modified fibers into Ad particles was assessed by Western blot analysis, which showed that the fibers did incorporate into virions very efficiently (Fig. 1). Based on these data, we conclude that the virions of these vectors have adequate structure and thus are suitable for tumor targeting experiments *in vivo*.

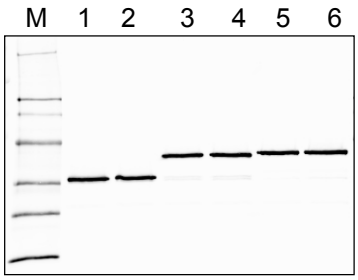


Figure 1. Western blot of purified Ad vectors. Aliquots of CsCl-purified viral preparations were fully denatured and fractionated by SDS-PAGE. After electroblotting, the samples were probed with the anti-Ad fiber monoclonal antibody 4D2. The fibers were detected using the fluorescently-labeled secondary antibody and a near-infrared scanner Odyssey.

M. protein mol. weight standards

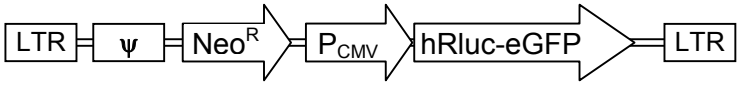
1. Ad5CMV-TL.F5
2. Ad5Flt-TL.F5
3. Ad5CMV-TL.$_{\Delta T}$PB40 1.7
4. Ad5Flt-TL.$_{\Delta T}$PB40 1.7
5. Ad5CMV-TL.$_{\Delta T}$PB40 1.12
6. Ad5Flt-TL.$_{\Delta T}$PB40 1.12

Task 4. Studies of the* in vivo *distribution and anti-tumor effect of the double-targeted Ad vectors.

In parallel, we prepared a target cell line for our *in vivo* studies. To facilitate the identification of the human tumor xenografts established in mice and also to allow for non-invasive monitoring of tumor growth, we made a derivative of human prostate carcinoma cell line LNCaP stably expressing a dual-modality reporter gene.

To this end, a recombinant replication-deficient retrovirus vector containing an open reading frame of *Renilla* luciferase (hRLuc) (Promega) fused with the enhanced green fluorescent protein (eGFP), was assembled within a commercial retroviral backbone, pLEGFP-N1 (BD Biosciences). This cloning put the dual reporter gene under transcriptional control of the cytomegalovirus (CMV) promoter. The resultant retro-vector was designated pLhRLuc-eGFP (Fig. 2).

Figure 2. Schematic representation of LhRLuc-EGFP genome. LTR – long terminal repeat, ψ - packaging signal, NeoR – G418 resistance gene, P_{CMV} – cytomegalovirus promoter, hRluc-eGFP – dual-reporter coding sequence.



This retro-vector was packaged by transfection of 293/GPG cells (5) that yielded pseudo-typed, pan-tropic virions decorated with the protein G of the vesicular stomatitis virus (VSV-G). The rescued vector was then used to transduce LNCaP cells and the stably transduced clones were selected using resistance to G418 as a selection marker (Neo^R).

Since the expression of the G418 resistance gene by this vector is not linked to the expression of the reporter transgene, an additional selection for GFP-producing cells was done using preparative cell sorting by flow cytometry. Two sequential sorting procedures resulted in a brightly fluorescent polyclonal cell line, LNCaP/hRluc-eGFP (Fig. 3).

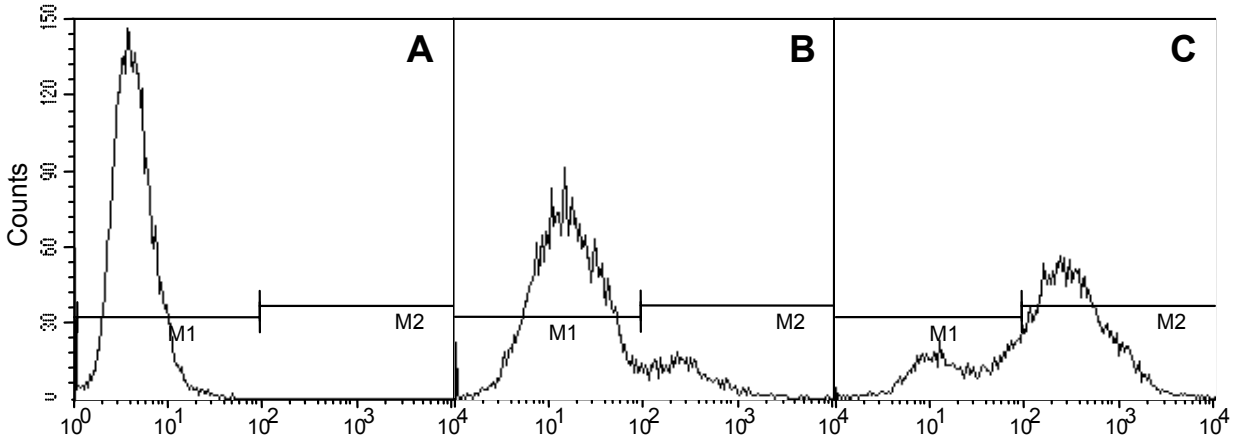


Figure 3. EGFP fluorescence in stably transduced LNCaP cells. Flow cytometry of the parental LNCaP cells (A), LNCaP cells after retroviral transduction (B), and polyclonal line LNCaP/hRluc-GFP obtained after two rounds of selection by cell sorting (C).

Analysis of Rluc activity in these cells (live and cell lysates) - confirmed a very high level of luciferase expression (Fig. 4). Therefore, if the intensity of GFP fluorescence of the LNCaP/Rluc-GFP-derived tumor xenografts in mice proves to be insufficient for tumor detection, visualization of the tumors will be done by bioluminescence-based imaging of Rluc activity.

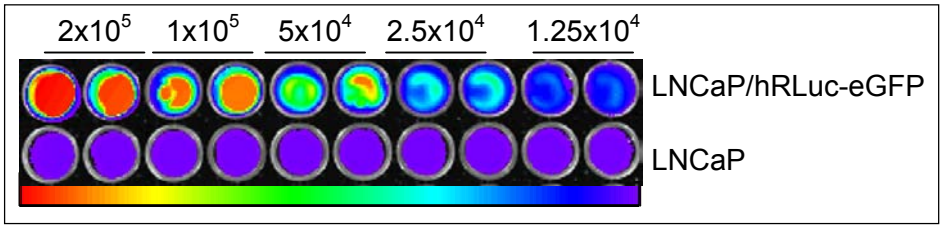


Figure 4. Bioluminescence measured in LNCaP cells using optical imaging. Cells, either parental LNCap or retrovirus-transduced, sorted LNCaP/Rluc-eGFP, were seeded in the wells of a 96-well plate in the amounts shown above the wells (cell/well) and after attachment were incubated with coelenterazine at 1μg/ml and imaged using the IVIS200 imaging system (Xenogen). Pseudo-color scale below the image shows the luminescence intensity from 10^6 photon/sec/cm^2/sr (purple), to $3.8x10^7$ photon/sec/cm^2/sr (red). According to this measurement, the photon output in LNCaP/hRLuc-EGFP cells equals $1.8x10^3$ photon/cell/sec.

After this characterization had been completed, this selected polyclonal line was expanded, and a frozen stock was made.

In addition, cell sorting was used to isolate individual clones of Rluc-GFP-producing LNCaP cells from this polyclonal line. Three brightly fluorescent clones have survived the selection and are currently in an expansion phase (Fig. 5). These clones may be used in our *in vivo* studies in case we encounter difficulties with either growing or detecting xenografts made with polyclonal line.

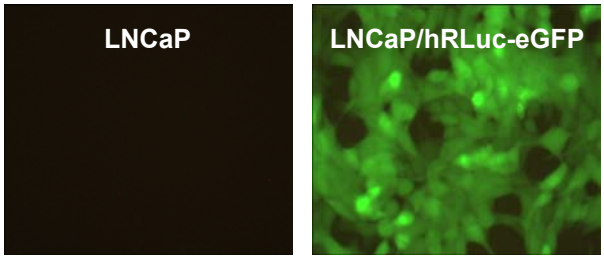


Figure 5. Fluorescent images of the parental LNCaP cells and a monoclonal cell line LNCaP/hRLuc-eGFP.

KEY RESEARCH ACCOMPLISHMENTS:

- A panel of recombinant Ad vectors expressing a dual-function transgene has been generated. The viruses have been amplified to the scale necessary for subsequent *in vivo* studies. The identities of these vectors have been confirmed by Western blotting and genome sequencing.

- A recombinant retrovirus vector expressing a dual-mode reporter gene (Renilla luciferase and green fluorescent protein) has been designed and produced in a pan-tropic configuration.

- Dual reporter-expressing poly- and monoclonal derivatives of the target cell line, which will be used for establishing tumor xenografts in mice, have been produced. Expression of the reporter in these lines was tested and found adequate for the *in vivo* imaging studies.

REPORTABLE OUTCOMES: N/A.

CONCLUSIONS:

In Year 3 the experimental work in the project continued along the lines of the original Statement of Work. However, the research program has not been finalized at the time of this report.

In addition to the previously reported delays (caused by our relocation to a new institution and additional experiments that we conducted in Year 2), the completion of the work was further delayed in Year 3. The reason for this was our findings in another on-going project that suggested a flaw in the strategy, which we used to design our vectors. Based on these findings, we re-evaluated the original vector design strategy and chose to remake the vectors. In our opinion, it was critically imortant to correct that error before the start of the *in vivo* experiments that are very time-, money-, and labor-consuming. Otherwise, we would most likely reach a point of no return few months later, whereby we would have neither time, nor resources to correct this mistake and redo the final part of the project.

As a result of this extra work on vector redesign, now we have a panel of vectors that have a much higher chance to function *in vivo* as expected.

In addition, we have developed cell lines (poly- and monoclonal) suitable for the *in vivo* validation of targeting capabilities of the designed Ad vectors. These lines have been made to produce two types of imaging reporters, the feature that will facilitate visualization of tumor xenografts and monitor their growth and regression.

Therefore, all the reagents necessary to do the *in vivo* work have been prepared and characterized. We are ready to do that work now.

As a negative result of the extra vector design and manufacturing work (above), we have not been able to eliminate the previously accumulated delay and, on the contrary, an even more substantial delay has been created. This could not be helped even by the addition of an extra lab scientist to our team (Ms Marine Kojanyan, a Research Assistant, whose position is supported by the PI's start-up funds, has been working on this project since joining our group in February 2006). Despite these delays, the project will be completed in its entirety as planned, because a no cost 12 months extension (through 01-31-08) has been granted.

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